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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/00, C12P 21/08, C12N 15/12, C07K 14/47, C12N 15/11, C07K 7/06, G01N 33/68, C12N 5/10

A2

(11) International Publication Number: **WO 95/23855**

(43) International Publication Date: 8 September 1995 (08.09.95)

(21) International Application Number: PCT/US95/02521

(22) International Filing Date: 1 March 1995 (01.03.95)

(30) Priority Data:
08/204,740 2 March 1994 (02.03.94) US

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(81) Designated States: AU, CA, JP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GENES AND GENETIC ELEMENTS ASSOCIATED WITH CONTROL OF NEOPLASTIC TRANSFORMATION IN MAMMALIAN CELLS

(57) Abstract

The invention provides genetic suppressor elements that confer the transformed phenotype of malignant mammalian cells upon untransformed cells, methods for identifying and obtaining such elements, methods for isolating and identifying genes corresponding to such elements, and methods of using such elements. The invention also provides genes corresponding to the GSEs of the invention.

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GENES AND GENETIC ELEMENTS ASSOCIATED WITH CONTROL OF NEOPLASTIC TRANSFORMATION IN MAMMALIAN CELLS

BACKGROUND OF THE INVENTION

1. Field of The Invention

The invention relates to genes and genetic suppressor elements associated with the control of neoplastic transformation of mammalian cells. More particularly, the invention relates to methods for identifying such genes and genetic suppressor elements as well as to uses for such genes and genetic suppressor elements. The invention specifically provides genetic suppressor elements derived from genes associated with the transformed phenotype of mammalian cells, and therapeutic and diagnostic uses related thereto. The invention also provides genes associated with the control of neoplastic transformation of mammalian cells.

2. Summary Of The Related Art

Cancer remains one of the leading causes of death in the United States. Clinically, a broad variety of medical approaches, including surgery, radiation therapy and chemotherapeutic drug therapy are currently being used in the treatment of human cancer (*see* the textbook CANCER: Principles & Practice of Oncology, 2d Edition, De Vita *et al.*, eds., J.B. Lippincott Company, Philadelphia, PA, 1985). However, it is recognized that such approaches continue to be limited by a fundamental lack of a clear understanding of the precise cellular bases of malignant transformation and neoplastic growth.

The beginnings of such an understanding of the cellular basis of malignant transformation and neoplastic growth have been elucidated over the last ten years. Growth of normal cells is now understood to be regulated by a balance of growth-promoting and growth-inhibiting genes, known as proto-oncogenes and tumor suppressor genes, respectively. Proto-oncogenes are turned into oncogenes by regulatory or structural mutations that increase their ability to stimulate uncontrolled cell growth. These mutations are therefore manifested as dominant (e.g. mutant RAS genes) or co-dominant (as in the case of amplification of oncogenes such as N-MYC or HER2/NEU) (*see* Varmus, 1989, "A historical overview of oncogenes", *in*

Oncogenes and the Molecular Origin of Cancer, Weinberg, ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., pp. 3-44).

Dominant and co-dominant genes can be effectively identified and studied using many different techniques based on gene transfer or on selective isolation of amplified or overexpressed DNA sequences (Kinzler *et al.*, 1987, *Science* 236: 70-73; Schwab *et al.*, 1989, *Oncogene* 4: 139-144; Nakatani *et al.*, *Jpn. J. Cancer Res.* 81: 707-710). Expression selection has been successfully used to clone a number of cellular oncogenes. The dominant nature of the oncogenes has facilitated the analysis of their function both *in vitro*, in cell culture, and *in vivo*, in transgenic animals. Close to fifty cellular oncogenes have been identified so far (Hunter, 1991, *Cell* 64: 249-270).

It is likely, however, that there are at least as many cancer-associated genes that are involved in suppression rather than induction of abnormal cell growth. This class of genes, known as anti-oncogenes or tumor suppressors, has been defined as comprising "genetic elements whose loss or inactivation allows a cell to display one or another phenotype of neoplastic growth deregulation" by Weinberg (1991, *Science* 254: 1138-1146). Changes in a tumor suppressor gene that result in the loss of its function or expression are recessive, because they have no phenotypic consequences in the presence of the normal allele of the same gene. The recessive nature of mutations associated with tumor suppressors makes such genes very difficult to analyze or identify by gene transfer techniques and explains why oncogene research is far more advanced than studies of tumor suppressors.

In normal cells, tumor suppressor genes may participate in growth inhibition at different levels, from the recognition of a growth inhibiting signal and its transmission to the nucleus, to the induction (or inhibition) of secondary response genes that finally determine the cellular response to the signal. The known tumor suppressor genes have indeed been associated with different steps of the regulatory pathway. Thus, the DCC and ErbA genes encode receptors of two different classes (Fearon *et al.*, 1990, *Science* 247: 49-56; Sap *et al.*, 1986, *Nature* 324: 635-640; Weinberger *et al.*, 1986, *Nature* 324: 641-646). The gene NF-1 encodes a polypeptide that resembles *ras*-interacting proteins, that are members of the signaling pathway (Xu *et al.*, 1990, *Cell* 62: 599-608; Ballester *et al.*, 1990, *Cell* 62: 851-859;

Buchberg *et al.*, 1990, *Nature* 347: 291-294; Barbacid, 1987, *Ann. Rev. Biochem.* 56: 779-827). p53, RB and WT genes encode nuclear regulatory proteins (Fields *et al.*, 1990, *Science* 249: 1046-1049; Raycroft *et al.*, 1990, *Science* 249: 1049-1051; Kern *et al.*, 1991, *Oncogene* 6: 131-136; O'Rourke *et al.*, 1990, *Oncogene* 5: 1829-1832; Kern *et al.*, 1991, *Science* 252: 1708-1711; Lee *et al.*, 1987, *Nature* 329: 642-645; Friend *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84: 9059-9063; Call *et al.*, 1990, *Cell* 60: 509-520; Gessler *et al.*, 1990, *Nature* 343: 774-778).

Two approaches have been previously used for cloning tumor suppressor genes. The first approach is based on isolating the regions associated with nonrandom genetic deletions or rearrangements observed in certain types of tumors. This approach requires the use of extremely laborious linkage analyses and does not give any direct information concerning the function of the putative suppressor gene (Friend *et al.*, 1991, *Science* 251: 1366-1370; Viskochil *et al.*, 1990, *Cell* 62: 187-192; Vogelstein *et al.*, 1988, *N. Engl. J. Med.* 319: 525-532). In fact, among numerous observations of loss of heterozygosity in certain tumors (Solomon *et al.*, 1991, *Science* 254: 1153-1160; LaForgia *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88: 5036-5040; Trent *et al.*, 1989, *Cancer Res.* 49: 420-423), there are only a few examples where the function of the affected gene is understood. In two of these rare cases the gene function was identified using another method, analysis of dominant negative mutant proteins (Herskowitz, 1987, *Nature* 329: 219-222).

Specifically, the tumor suppressor genes *erbA* and *p53* were first discovered as altered forms which encoded mutant proteins (Sap *et al.*, 1986, *ibid.*; Weinberger *et al.*, 1986, *ibid.*; Raycroft *et al.*, 1990, *ibid.*; Milner *et al.*, 1991, *Molec. Cell. Biol.* 11: 12-19). These altered genes were initially classified as oncogenes, since they induced cell transformation when transfected alone or in combination with other oncogenes (*ras* in the case of *p53* and *erbB* in the case of *erbA*; *see* Eliyahu *et al.*, 1984, *Nature* 312: 646-649; Parada *et al.*, 1984, *Nature* 312: 649-651; Graf & Beug, 1983, *Cell* 34: 7-9; Damm *et al.*, 1989, *Nature* 339: 593-597). Later, however, it was recognized that both of these "oncogenes" acted by interfering with the normal function of the corresponding wild-type genes. Thus, the oncogenic mutant p53 protein forms functionally inactive complexes with the wild-type protein; such complexes fail to provide the normal negative regulatory function of the p53 protein

(Herskowitz, 1986, *ibid.*; Milner *et al.*, 1991, *ibid.*; Montenarh & Quaiser, 1989, *Oncogene* 4: 379-382; Finlay *et al.*, 1988, *Molec. Cell. Biol.* 8: 531-539). The oncogene *erbA*, found in chicken erythroblastosis virus, is a mutant version of the chicken gene for thyroid hormone receptor, the transcriptional regulatory protein which participates in the induction of erythroid differentiation (Damm *et al.*, 1989, *ibid.*; Damm *et al.*, 1987, *EMBO J.* 6: 375-382). The mutant *erbA* protein blocks the function of the wild-type receptor by occupying its specific binding sites in the DNA (Sap *et al.*, 1989, *Nature* 340: 242-244).

Thus, naturally arising dominant negative mutants not only allowed the identification of the corresponding tumor suppressor genes but also served as tools for their functional analysis. Such natural tools for recessive gene identification seem to be rare, however, limiting the utility of this approach for the discovery of new tumor suppressor genes.

The discovery and analysis of new recessive genes involved in neoplastic transformation may be greatly accelerated through the use of genetic suppressor elements (GSEs), derived from such genes and capable of selectively suppressing their function. GSEs are dominant negative factors that confer the recessive-type phenotype for the gene to which the particular GSE corresponds. Recently, some developments have been made in the difficult area of isolating recessive genes using GSE technology. Roninson *et al.*, U.S. Patent No. 5,217,889 (issued June 8, 1993) teach a generalized method for obtaining GSEs (*see also* Holzmayer *et al.*, 1992, Nucleic Acids Res. 20: 711-717). Gudkov *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90: 3231-3235 teach isolation of GSEs from topoisomerase II cDNA that induce resistance to topoisomerase II-interactive drugs. Co-pending U.S. Patent Applications Serial No. 08/033,986, filed March 3, 1993, and Serial No. 08/177,571, filed January 5, 1994, disclosed the discovery by the present inventors of the novel and unexpected result that GSEs isolated from RNA of cells resistant to the anticancer DNA damaging agent, etoposide, include a GSE encoding an antisense RNA homologous to a portion of a kinesin heavy chain gene. Additionally, co-pending U.S. Patent Application Serial No. 08/033,986 disclosed two other GSEs from previously-unknown genes, the expression of said GSEs conferring etoposide resistance on mammalian cells. Co-pending U.S. Patent Application Serial No.

08/199,900, filed February 22, 1994, disclosed GSEs from previously-unknown genes, the expression of said GSEs conferring cisplatin resistance on mammalian cells.

These results further underscored the power of the GSE technology developed by these inventors to elucidate recessive gene-mediated biological phenomenon involving unexpected mechanisms, including drug resistance in cancer cells, thereby providing the opportunity and the means for overcoming drug resistance in cancer patients. This technology has now been applied to isolating and identifying GSEs that confer the transformed phenotype of malignant mammalian cells in previously untransformed cells expressing such GSES, and for isolating and identifying genes associated with the transformed phenotype.

BRIEF SUMMARY OF THE INVENTION

The invention provides genetic suppressor elements (GSEs) that are random fragments derived from genes associated with the transformed phenotype of malignant mammalian cells, and that confer the transformed phenotype upon cells expressing such GSEs. The invention is based in part on the discoveries disclosed in co-pending U.S. Patent Applications, Serial No. 08/033,086, filed March 3, 1993, Serial No. 08/177,157, filed January 5, 1994, and Serial No. 08/199,900, filed February 22, 1994, incorporated by reference, providing a method for identifying and isolating GSEs that confer resistance to chemotherapeutic drugs upon cells expressing such GSEs.

In a first aspect, the invention provides a method for identifying GSEs that confer the transformed phenotype on cells expressing the GSEs. This method utilizes selection of cells that harbor clones from a random fragment expression library derived from total cDNA derived from normal cells, preferably normal mouse or human fibroblasts, and subsequent rescue of library inserts from immortalized, morphologically-transformed or frankly tumorigenic cells. In a second aspect, the invention provides a method for identifying and cloning genes that are associated with the transformed phenotype of malignant mammalian cells, and also provides the genes themselves. This method comprises the steps of screening a full length cDNA library with a GSE that confers the transformed phenotype upon cells (or,

alternatively, with an oligonucleotide or polynucleotide constituting a portion of such a GSE) and determining the nucleotide sequence of the cDNA insert of any positive clones obtained. Alternatively, the technique of "anchored PCR" (*see* Example 3 *below*) can be used to isolate cDNAs corresponding to transformed phenotype-conferring GSEs. Also embodied in this aspect of the invention is isolation of genomic DNA encoding genes associated with the transformed phenotype, for example from genomic DNA libraries. In a third aspect, the invention provides a diagnostic assay for characterizing transformed cells, particularly human tumor cells, that express the transformed phenotype due to the absence of expression or underexpression of a particular gene. This diagnostic assay comprises measuring, preferably quantitatively, the level of expression of the particular gene product by a particular tumor cell sample to be tested, compared with the level of expression in normal, untransformed cells. One feature of this aspect of the invention is the development of antibodies specific for proteins whose underexpression or absence of expression is associated with the transformed phenotype in malignant mammalian, most preferably malignant human, cells. Such antibodies have utility as diagnostic agents for detecting tumor cells in biopsy or other tissue samples, and in characterizing the nature and degree of expression of the transformed phenotype in such cells. In a fourth, the invention provides a starting point for *in vitro* drug screening and rational design of pharmaceutical products that are useful against tumor cells, *i.e.*, are anticancer agents. By examining the structure, function, localization and pattern of expression of genes associated with the transformed phenotype, strategies can be developed for creating pharmaceutical products that will selectively kill or inhibit the growth of such cells, in which such genes are either not expressed or underexpressed. Also provided by the invention are cultures of mammalian cells which express the transformed phenotype-conferring GSEs of the invention and are transformed thereby. Such cells are useful for determining the physiological and biochemical basis for malignant mammalian cell transformation. Such cells also have utility in the development of pharmaceutical and chemotherapeutic agents for selectively killing or inhibiting the growth of such cells, and thus are ultimately useful in establishing improved chemotherapeutic protocols to more effectively treat neoplastic disease.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of the adaptor used in cDNA cloning. The nucleotide sequences are shown for the ATG-sense (SEQ.ID.No.:1) and ATG-antisense (SEQ.ID.No.:2) strands of the adaptor.

Figure 2 shows the structure of the pLNCX vector used in cDNA cloning.

Figures 3A and 3B show a scheme for selection of immortalizing GSEs in MEF cells from a random fragment expression library (RFEL) from mouse NIH 3T3 cell cDNA. Figure 3A illustrates selection of such GSEs *via* one round of selection for cells that survive crisis; Figure 3B shows a scheme for re-selection and enrichment of immortalizing GSEs from populations of immortalized MEFs produced according to the scheme shown in Figure 3A.

Figure 4 shows polyacrylamide gel electrophoretic analysis of PCR fragments comprising MEF immortalizing GSE.

Figure 5 shows the nucleotide sequence of the Tr6-GSE (SEQ ID No.:3).

Figures 6A and 6B show the results of an experiment demonstrating that Tr6-GSE (SEQ ID No.:3) is capable of conferring the morphologically transformed phenotype on both Swiss 3T3 cells and MEF cells (Figure 6A), and is also capable of immortalizing MEF cells in which spontaneous immortalization is suppressed by expression of an exogenously-introduced p53 gene (Figure 6B).

Figure 7 shows a scheme for selecting morphological transformation-conferring GSEs.

Figures 8A and 8B show the results of an experiment in which rescued transforming GSE-carrying retroviruses were used to re-infect fresh NIH 3T3 cells. Figure 8A shows the results of selection of cells infected with virus from foci 24, 25, and 26 for G418 resistance (as a measure of infection efficiency) and morphological transformation in media supplemented with 5% FCS; Figure 8B shows the results of PCR analysis of retroviral inserts from genomic DNA of morphologically transformed foci.

Figure 9 shows the nucleotide sequence of the SAHH-GSE (SEQ ID No.:4).

Figure 10 shows a comparison between the nucleotide sequence of SAHH-GSE (SEQ ID No.:4; *upper sequence*) and the human S-adenosylhomocysteine hydrolase mRNA sequence (SEQ ID No.:5; *lower sequence*).

Figure 11 shows a comparison between the amino acid sequence of the peptide encoded by the SAHH-GSE (SEQ ID No.:6; *upper sequence*) and the human S-adenosylhomocysteine hydrolase protein amino acid sequence (SEQ ID No.:7; *lower sequence*).

Figure 12 shows the nucleotide sequence of the Tr19-GSE (SEQ ID No.:8).

Figures 13A-13C show the results of an experiment demonstrating that SAHH-GSE was capable of conferring both immortalization and morphological transformation on MEF cells (Figure 13A); that Tr19-GSE is capable of immortalizing MEF cells (Figure 13B); and that both the SAHH-GSE and an anti-*khcs* GSE could immortalize MEF cells, but only the SAHH-GSE could morphologically transform MEF cells (Figure 13C).

Figure 14 shows a scheme for selecting tumorigenic GSEs.

Figure 15 polyacrylamide gel electrophoretic analysis of PCR fragments comprising tumorigenic GSEs.

Figure 16 shows the nucleotide sequence of the Tr22-GSE (SEQ ID No.:9).

Figure 17 shows the nucleotide sequence of the 1bb1-GSE (SEQ ID No.:10).

Figure 18 shows a comparison between the nucleotide sequence of the 1bb1-GSE (SEQ ID No.:10; *upper sequence*) and the P120 human nucleolar antigen gene sequence (SEQ ID No.:11; *lower sequence*).

Figure 19 shows a comparison between the amino acid sequence of the peptide encoded by the 1bb1-GSE (SEQ ID No.:12; *upper sequence*) and a portion of the P120 human nucleolar antigen protein amino acid sequence (SEQ ID No.:13; *lower sequence*).

Figure 20 shows the results of a focus-formation assay using infection of Swiss 3T3 cells with retrovirus carrying the 1bb1-GSE (SEQ ID No.:10).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to means for identifying specific gene functions that are associated with the transformed phenotype of malignant mammalian cells. The invention provides genetic suppressor elements (GSEs), the expression of such GSEs conferring the transformed phenotype on untransformed fibroblast cells. The invention further provides methods for identifying such GSEs, as well as methods for their use. For purposes of this invention, the terms "the transformed phenotype of malignant mammalian cells" and "the transformed phenotype " are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

In a first aspect, the invention provides a method for identifying GSEs that confer upon untransformed cells the transformed phenotype of malignant mammalian cells. The GSEs identified by this method will be homologous to a gene that is associated with the transformed phenotype of malignant mammalian cells. For purposes of the invention, the term "homologous to a gene" has two different meanings, depending on whether the GSE acts through an antisense or antigene mechanism, or through a mechanism of interference at the protein level. In the former case, a GSE that is an antisense or antigene oligonucleotide or polynucleotide is homologous to a gene if it has a nucleotide sequence that hybridizes under physiological conditions to the gene or its mRNA transcript by Hoogsteen or Watson-Crick base-pairing. In the latter case, a GSE that interferes with a protein molecule is homologous to the gene encoding that protein molecule if it has an amino acid sequence that is the same as that encoded by a portion of the gene encoding the protein, or that would be the same, but for conservative amino acid substitutions. In either case, as a practical matter, whether the GSE is homologous to a gene is determined by assessing whether the GSE is capable of inhibiting or reducing the function of the gene.

The method according to this aspect of the invention comprises the step of screening a total cDNA or genomic DNA random fragment expression library

phenotypically to identify clones that confer the transformed phenotype on untransformed recipient cells. Preferably, the library of random fragments of total cDNA or genomic DNA is cloned into a retroviral expression vector. In this preferred embodiment, retrovirus particles containing the library are used to infect cells and the infected cells are tested for their ability to exhibit the transformed phenotype, for example, by exhibiting the ability to grow past "crisis" in *vitro* culture, or to grow in a manner that is recognized as being morphologically-transformed, or to grow in semisolid media, such as soft agar or agarose, or in methylcellulose, or by frankly tumorigenic growth *in vivo* in an animal. Preferably, the inserts in the library will range from about 100 bp to about 700 bp and more preferably, from about 200 bp to about 500 bp in size. Most preferably, the random fragment library will be a normalized library containing roughly equal numbers of clones corresponding to each gene expressed in the cell type from which it was made, without regard for the level of expression of any gene. However, normalization of the library is unnecessary for the isolation of GSEs that are homologous to abundantly or moderately expressed genes. Once a clonal population of cells that exhibit the transformed phenotype has been isolated, the library clone encoding the GSE is rescued from the cells. At this stage, the insert of the expression library may be tested for its nucleotide sequence. Alternatively, and preferably, the rescued library clone may be further tested for its ability to confer the transformed phenotype in additional transfection or infection and selection assays, prior to nucleotide sequence determination. Determination of the nucleotide sequence, of course, results in the identification of the GSE. This method is further illustrated in Examples 1 and 2.

In a second aspect, the invention provides a method for identifying and cloning genes that are associated with control of neoplastic growth in mammalian cells, as well as the genes derived by this method. This is because GSEs, or portions thereof, can be used as probes to screen full length cDNA or genomic libraries to identify their gene of origin. Alternatively, the technique of "anchored PCR" (*see* Example 3 *below*) can be used to isolate cDNAs corresponding to transformed phenotype-conferring GSEs. It will be recognized that the genes associated with control of neoplastic transformation in mammalian cells are

sufficiently evolutionarily conserved that the GSEs provided by the invention, or the genes corresponding to such GSEs, can be used as probes to isolate genes corresponding to such neoplastic growth-associated GSEs from any mammalian species, including man.

In some cases, genes that are associated with the transformed phenotype will turn out to be quite surprising. For example, GSEs that have been found to be capable of conferring the transformed phenotype upon untransformed cells include GSEs derived from the mouse homolog of the human P120 nucleolar antigen gene, and the gene for *S*-adenosyl homocysteine hydrolase, as well as from three GSEs from previously unidentified genes. In addition, a GSE derived from a mouse kinesin gene and associated with etoposide resistance has been previously discovered to be capable of conferring cell culture growth immortalization on mouse embryo fibroblasts (MEF) and normal human fibroblasts, as disclosed in co-pending U.S. Patent Applications, Serial No. 08/177,154, filed January 5, 1994, and Serial No. 08/033,086, filed March 3, 1993. The method according to this aspect of the invention therefore also provides valuable information about the genetic basis for senescence. The method according to this aspect of the invention and its use for studying genes identified thereby and their cellular effects are further illustrated in Example 3.

In a third aspect, the invention provides a diagnostic assay for characterizing transformed cells, particularly human tumor cells, that express the transformed phenotype due to the absence of expression or underexpression of a particular gene. By using the methods according to the first and second aspects of the invention such a gene is identified and cloned. To determine whether absence of expression or underexpression of such a gene is a naturally occurring, and thus medically significant basis for neoplastic growth and cancer, human tumor cells are assessed for their level of expression of the particular gene of interest. Absence of expression or significantly reduced expression, relative to expression in normal tissues that give rise to the tumor, would then be correlated with the natural history of the particular cancer, including cell and tissue type, incidence, invasiveness, capacity to metastasize, and other relevant properties of the particular tumor. Accordingly, such reduced or absent expression can be the basis for a diagnostic assay for the presence

and extent of tumorigenic cells in a tissue sample. Malignant transformation and neoplastic growth as the result of over-expression of a gene is also detectable using similar diagnostic assays provided by the invention. A first embodiment of a diagnostic assay according to this aspect of the invention utilizes an oligonucleotide or oligonucleotides that is/are homologous to the sequence of the gene for which expression is to be measured. In this embodiment, RNA is extracted from a tissue or tumor sample, and RNA specific for the gene of interest is quantitated by standard filter hybridization procedures, an RNase protection assay, or by quantitative cDNA-PCR (*see* Noonan *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87: 7160-7164). In a second embodiment of a diagnostic assay according to this aspect of the invention, antibodies are raised against a synthetic peptide having an amino acid sequence that is identical to a portion of the protein that is encoded by the gene of interest. These antibodies are then used in a conventional quantitative immunoassay (*e.g.*, RIA or immunohistochemical assays) to determine the amount of the gene product of interest present in a sample of proteins extracted from the tumor cells to be tested, or on the surface or at locations within the tumor cells to be tested. In a third embodiment, an enzymatic activity that is a property of a gene associated with neoplastic transformation of cancer cells can be used to measure whether the gene encoding said protein is over- or under-expressed in the cancer cells.

In a fourth aspect, the invention provides a starting point for *in vitro* drug screening and rational design of pharmaceutical products that can counteract tumorigenicity and neoplastic growth by tumor cells *in vivo*. In this regard, the invention provides cultures of mammalian cells which express the transformed phenotype-conferring GSEs of the invention and are immortalized and/or transformed thereby. Included within this aspect of the invention are cell cultures that are representative of almost any tissue or cell type. Such cells are useful for determining the physiological and biochemical basis for malignant transformation of mammalian cells, as well as for screening pharmaceutical and chemotherapeutic agents for killing or selectively inhibiting the growth os such transformed cells. Identification of such agents would lead to the development of improved chemotherapeutic protocols to more effectively treat neoplastic disease.

The protein sequence encoded by genes from which the GSEs were derived can be deduced from the cDNA sequence, and the function of the corresponding proteins may be determined by searching for homology with known genes or by searching for known functional motives in the protein sequence. If these assays do not indicate the protein function, it can be deduced through the phenotypic effects of the GSEs suppressing the gene. Such effects can be investigated at the cellular level, by analyzing various growth-related, morphological, biochemical or antigenic changes associated with GSE expression. The GSE effects at the organism level can also be studied by introducing the corresponding GSEs as transgenes in transgenic animals (*e.g.* mice) and analyzing developmental abnormalities associated with GSE expression. The gene function can also be studied by expressing the full-length cDNA of the corresponding gene, rather than a GSE, from a strong promoter in cells or transgenic animals, and studying the changes associated with overexpression of the gene.

Full-length or partial cDNA sequences can also be used to direct protein synthesis in a convenient prokaryotic or eukaryotic expression system, and the produced proteins can be used as immunogens to obtain polyclonal or monoclonal antibodies. These antibodies can be used to investigate the protein localization and as specific inhibitors of the protein function, as well as for diagnostic purposes. In particular, antibodies raised against a synthetic peptide encoded by the sequence of the GSEs Tr6, Tr19 and Tr22, or the corresponding region of the P120 nucleolar antigen gene or the SAHH gene should be particularly useful (*see* Examples 2 and 3 and Figures 5, 9-11, & 15-18).

Understanding the biochemical function of a gene involved in malignant transformation of mammalian cells is also likely to suggest pharmaceutical means to stimulate or mimic the function of such a gene and thus augment the cytotoxic response to anticancer drugs. For example, if the gene encodes an enzyme producing a certain compound, such a compound can be synthesized chemically and administered in combination with cytotoxic drugs. If a pharmaceutical approach is not apparent from the protein function, one may be able to upmodulate gene expression at the level of transcription. This can be done by cloning the promoter region of the corresponding gene and analyzing the promoter sequence for the

presence of *cis* elements known to provide the response to specific biological stimulators. Such an approach is useful to replace the function of tumor-suppressor genes, for example, to restore the tumor-suppressing function of such genes that has been lost through mutation or other biological insult, resulting in neoplastic disease.

The most straightforward way to increase the expression of gene identified through the GSE approach, the loss of which results in malignant transformation of a cell no longer functionally expressing the gene, would be to insert a full-length cDNA for such a gene into a gene therapy expression vector, for example, a retroviral vector. Such a vector, in the form of a recombinant retrovirus, will be delivered to tumor cells *in vivo*, and, upon integration, would act to reduce or eliminate neoplastic growth of such cells. The selective delivery to tumor cells can be accomplished on the basis of the selectivity of retrovirus-mediated transduction for dividing cells. Alternatively, the selectivity can be achieved by driving the expression of the gene from a tissue- or tumor-specific promoter, such as, for example, the promoter of the carcinoembryonic antigen gene.

The protein structure deduced from the cDNA sequence can also be used for computer-assisted drug design, to develop new drugs that affect this protein in the same manner as the known anticancer drugs. The purified protein, produced in a convenient expression system, can also be used as the critical component of *in vitro* biochemical screen systems for new compounds with anticancer activity. In addition, mammalian cells that express tranformed phenotype-conferring GSEs according to the invention are useful for screening compounds for the ability to selectively kill or inhibit the neoplastic growth associated with down-regulation of the corresponding gene.

The following Examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

EXAMPLE 1

Generation of a Normalized Random Fragment cDNA Library in a Retroviral Vector and Introduction Into Virus-Packaging Cell Lines

A normalized cDNA population was prepared as described in co-pending U.S. Patent Application Serial No. 08/033,086, filed March 9, 1993, which is

incorporated by reference. Briefly, poly(A)$^+$ RNA was purified from total RNA extracted in equal amounts from exponentially-growing and quiescent, confluent monolayer cultures of mouse NIH 3T3 cells (Accession No. CRL 1658, American Type Culture Collection, Rockville, MD), an immortalized mouse cell line known to be useful in cellular transformation assays (*see* Shih *et al.*, 1979, *Proc. Natl. Acad. Sci. USA* 76: 5714-5718). To avoid over-representation of the 5'-end sequences in a randomly primed cDNA population, RNA was fragmented by boiling for 5 minutes to an average size of 600-1000 nucleotides. These RNA fragments were then used for preparing randomly primed double-stranded cDNA. This randomly primed cDNA was then ligated to a synthetic adaptor providing ATG codons in all three possible reading frames and in a proper context for translation initiation (*see* Figure 1). The structure of the adaptor determined its ligation to the blunt-ended fragments of the cDNA in such a way that each fragment started from initiation codons independently from its orientation. The ligated mixture was amplified by PCR, using the "sense" strand of the adaptor as a PCR primer, in twelve separate reactions that were subsequently combined, in order to minimize random over-or under-amplification of specific sequences and to increase the yield of the product. The PCR-amplified mixture was then size-fractionated by electrophoresis in a 6% polyacrylamide gel, and fragments ranging in size from approximately 200-500 basepairs (bps) were selected for further manipulations.

For normalization, the cDNA preparation was denatured and reannealed, using the following time-points for reannealing: 0, 24, 48, 72, 96 and 120 hours. The single-stranded and double-stranded DNAs from each reannealed mixture were then separated by hydroxyapatite chromatography. These DNA fractions from each time point of reannealing were PCR-amplified using adaptor-derived primers and analyzed by slot blot hybridization with probes corresponding to genes expressed at different levels in human cells. α-tubulin and c-*myc* probes were used to represent highly-expressed genes, adenosine deaminase and topoisomerase-II (using separate probes for the 5' and 3' ends of the latter cDNA) probes were used to represent intermediately-expressed genes, and a c-*fos* probe was used to represent low-level expressed genes. The fraction that contained similar proportions of high-, medium- and low-expressed genes was used for the library preparation.

The normalized cDNA preparation was cloned into a *Cla*I site of the MoMLV-based retroviral vector pLNCX, which carries the *neo* (G418 resistance) gene, expressed under the transcriptional control of the promoter contained in the retroviral long terminal repeat (LTR), and which expresses the cDNA insert sequences from a cytomegalovirus (CMV)-derived promoter (*see* Figure 2 and Miller and Rosman, 1989, Biotechniques 7: 980-986). pLNCX contains translation termination codons in all three reading frames within 20 bp downstream of the cloning site. To generate a representative-size library for GSE selection, this ligation mixture was divided into five portions and used to transform *E. coli* in 5 separate electroporation experiments, using conventional techniques and standard conditions for electroporation (*see* Sambrook *et al.*, 1992, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The transformed bacteria were plated on a total of 500 agar plates (150mm in diameter) and the plasmid produced (18mg total) was isolated from the colonies washed off the agar. A total of approximately 5 x 10^7 clones were obtained, more than 60% of which carried inserts of normalized cDNA, as estimated by PCR amplification of 50 randomly-picked colonies.

Plasmid DNA was used for *in vivo* selection of GSEs capable of conferring a transformed phenotype of appropriate cells as discussed in Example 2 below. The plasmid library prepared as described above was converted into a mixture of retroviral particles by transfection into twenty P150 culture plates containing a 1:1 mixture of ecotropic and amphotropic packaging cells (derived from NIH 3T3 cells; *see* Markowitz *et al.*, 1988, *Virology* 167: 400-406), the cells having been seeded the day before transfection at a density of 1.5 x 10^6 cells per plate. 15μg of random fragment retroviral library (RFRL) plasmid DNA were transfected per P150 plate. The retrovirus-containing cell culture supernatant was collected every 12 hours over three days post-transfection and purified by filtration through 0.22μm membranes.

EXAMPLE 2

Introduction Of A Retroviral Random Fragment Library Into Mouse Fibroblast Cells

The purified retrovirus-containing supernatant prepared according to Example 1 was used in each of three assays chosen to detect three distinct aspects of the transformed phenotype in mammalian cells. Selection of transforming GSEs required the use of suitable indicator cells capable of undergoing identifiable and selectable transformation-associated changes. Three different selection protocols for GSEs that induce phenotypic traits associated with neoplastic transformation were used. First, for selection of GSEs capable of immortalizing senescent cells, mouse embryonic fibroblasts were used as the indicator cell system. The other two selection protocols utilized three different types of immortalized mouse fibroblasts, each of which differ in transformation-associated traits, in order to select GSEs specific for different stages of neoplastic transformation. Two of these cell lines are subvariants of NIH 3T3 cells, and the third type of cells comprise several populations of Swiss 3T3 cells, newly established from spontaneously-transformed MEF cells. These latter cells were expected to contain multiple phenotypic variants which would be differentially susceptible to the effects of different GSEs, thereby increasing the number of different types of GSEs that could be detected. Some characteristic properties of each of the three types of immortalized cells are shown in Table I.

TABLE I

Cell Type	Rate of Spontaneous Focus Formation	Plating Efficiency	Tumorigenicity[a] 3 Weeks	Tumorigenicity[a] 6 weeks
NIH 3T3-HF	$2\text{-}5 \times 10^{-6}$	20-30%	0/6	5/6
NIH 3T3-LF	$< 1 \times 10^{-7}$	20-30%	0/6	0/6
Swiss 3T3	$< 1 \times 10^{-7}$	< 0.1%	N.T.	N.T.

[a] = Number of mice with tumors/Number of mice tested
N.T = not tested

A. Selection of GSEs Capable of Imm rtalizing M use Embryo Fibroblasts

GSE selection for the ability to immortalize senescent cells was carried out on cultures of mouse embryo fibroblast (MEF) cells infected with retroviral particles comprising the RFRL of Example 1, using a protocol depicted in Figures 3A and 3B. Primary MEF cultures were prepared from 11-day old Swiss Webster mouse embryos using a conventional trypsinization procedure. Cells were split every three-four days, with 2.5 x 10^6 cells plated per P150 culture plate at each passage, grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum. Additionally, about 5 x 10^6 cells were preserved after every second passage until the culture underwent senescence and "crisis", by freezing in a cryogenic protective solution at -70°C. For retroviral infection experiments, cells frozen 4 passages before crisis were thawed and grown in culture on 10 P150 plates at a density of 1 x 10^6 cells/plate. The thawed cells were infected with RFRL-derived retrovirus over 3 days, at 12 hour intervals, and MEFs were repeatedly infected with each collected supernatant. Each P150 plate was processed independently beginning with infection with the RFRL-derived retrovirus. The efficiency of infection was estimated by plating equal numbers of infected cells in the presence and absence of G418 for 5 days, at which time relative cell viability was measured using the MTT assay (*see* Pauwels *et al.*, 1988, *J. Virol. Meth.* 20: 309-321). Typical infections efficiencies obtained in such assays indicated that about 70% of the MEFs were infected with RFRL-derived retroviruses.

After the cell cultures overcame senescence and crisis, the surviving cells from each plate were fused with ecotropic packaging cells to rescue the virus, using polyethylene glycol as previously described in co-pending U.S. Patent Application Serial No. 08/199,900, filed on February 22, 1994. The complexity of the rescued virus population was estimated by PCR amplification of proviral inserts, using the oligonucleotide corresponding to the sense strand of the cloning adaptor as PCR primer (as shown in Figure 4). The PCR products from RFRL-derived retrovirus infected MEF cells initially formed a continuous smear of fragments 200-500 bps in length. As the cells proceeded through crisis, the complexity of the cDNA inserts decreased, and separate bands became visible (Figure 2).

The rescued viral preparations from post-crisis cells, containing the virus at relatively low titre ($\sim 10^4$/mL), were used to infect fresh populations of pre-crisis MEF cells, which were then allowed to go through crisis. The efficiency of these secondarily-infected cells was estimated by G418 selection before and after crisis; in several secondary selection experiments, the proportion of infected cells increased after crisis, suggesting enrichment for GSE-carrying cells. PCR analysis performed on cellular DNA from immortalized cells surviving this second round selection indicated the selection of several cDNA inserts, containing putative immortalization-conferring GSEs.

These inserts are each individually subcloned into the pLNCX retroviral vector and tested for the ability to immortalize MEF cells as shown in Figure 3B. MEFs that are two passages before crisis are infected by GSE-carrying viruses and then plated at low density (*e.g.*, 3 x 10^4 cells/100mm culture plate) and then fixed and stained two weeks after plating. The number of surviving colonies reflects the proportion of immortalized cells in the infected population.

B. Isolation of GSEs that Can Morphologically Transform Mouse Fibroblasts

To isolate GSEs capable of inducing morphological transformation of immortalized MEFs, immortalized MEF cells as described in subsection A above were used. Cells were plated into 10 P100 plates at a density of 2.5 x 10^6 cells/plate and maintained in DMEM/10% FCS for three weeks. 2-20 foci of morphologically-transformed cells appeared in each plate. Two foci were isolated and expanded by growth in culture. Cells from these expanded foci were then fused with packaging cells and the hybrid cells selected with G418 and used to rescue retroviral populations as described above. Viruses isolated in this way from the expanded foci were used to infect fresh Swiss 3T3 cells, and the infected cells were maintained in DMEM/5% FCS.

Viruses rescued from each of these two foci, isolated from one of the original plates of immortalized MEF cells, induced morphological transformation of Swiss 3T3 cells in two separate experiments. PCR analysis of the cDNA insert present in the transforming virus (termed Tr6-GSE), performed on genomic DNA isolated from four independent foci of transformed Swiss 3T3 cells, revealed a single insert band.

DNA from this band was re-cloned into the pLNCX vector and the nucleotide sequence determined using conventional techniques (*see* Sambrook *et al.*, *ibid.*). This clone was found to contain a 285 bp insert (shown in Figure 5), which showed no significant homology with known nucleic acid and protein sequences present in the National Center for Biotechnology Information database. The re-cloned Tr6-GSE-carrying retrovirus was efficient in inducing morphological transformation of NIH 3T3 cells and immortalized MEF (shown in Figure 6A). Infection of senescent MEF cells with this virus produced no significant increase in the number of immortalized cells, relative to background.

Tr6, however, was found to have an effect on MEF immortalization by a different assay. In this assay, MEF cells 2 passages from senescence were infected with LNCX, or LNCX carrying Tr6-GSE, or a retroviral construct carrying a full-length cDNA encoding the cellular tumor suppressor gene p53, or a combination of the p53 retrovirus and Tr6-GSE carrying retrovirus. MEF cells infected with the LNCX vector retrovirus produced a low background spontaneously-immortalized cells (Figure 6B). In contrast, MEF cells infected with the recombinant retrovirus carrying a full-length cDNA of the p53 tumor suppressor gene under conditions where all the cells were infected, failed to give rise to any immortalized colonies. However, when the same cells were infected under the same conditions with retroviruses carrying Tr6 and p53, immortalized colonies were formed (Figure 6B).

GSEs were also selected for the ability to induce morphological transformation of NIH 3T3 cells (shown in Figure 7). In these experiments, RFRL plasmid DNA was transfected into a 1:1 mixture of ecotropic and amphotropic virus-packaging cells. Retroviral particle-containing tissue culture media supernatant was collected at 24, 48 and 72h after infection and used for repeat infection of NIH 3T3 cells. The total amount of virus used for infection was estimated to be $> 10^7$ infectious units. Recipient NIH 3T3 cells were plated in ten P150 plates at a density of 1×10^6 cells/plate and incubated in DMEM/10% FCS. Four plates were infected with control virus containing no GSE insert, produced by transient transfection of packaging cells with the vector plasmid pLNCX, to estimate the rate of spontaneous (*i.e.*, non-GSE mediated) transformation in these cells.

The day after the last infection, a portion of the infected NIH 3T3 cells were frozen as described above, and another portion was split into 10 P150 culture plates at a density of 2 x 10^6 cells/plate and cultured in DMEM/5% FCS for two weeks. The efficiency of infection was evaluated by G418 selection; typically, at least 50% of the cells were found to be infected. Similar numbers of apparently transformed cells were observed in both the experimental and control plates (5-15 foci/plate, corresponding to 2.5-7.5 x 10^{-6} foci/cell). Individual foci were picked and expanded as described above, and virus rescued from each focus by fusion with ecotropic packaging cells. Fresh NIH 3T3 cells were infected with rescued retrovirus, and cells infected with 2/50 rescued virus populations were found to produce cell populations which showed altered growth properties, including reaching a much higher density in 5% serum (shown in Figures 8A and 8B). PCR analysis of genomic DNA from these populations showed that each of the two virus preparations inducing such altered cellular growth properties carried a single cDNA insert.

The two cDNA inserts carried by the transforming retroviruses isolated in this manner were sequenced and analyzed for homology with known nucleic acid and protein sequences present in the NCBI database. This analysis showed that one of the transforming viruses carried a 285 bp fragment corresponding to the beginning of the coding region of the cDNA encoding the enzyme *S*-adenosyl homocysteine hydrolase (SAHH), cloned in the sense orientation (shown in Figures 9-11). SAHH is known to be involved in many biochemical pathways, including methionine, cysteine and *S*-adenosylmethionine synthesis, the latter compound being the major source of methyl groups in methylation reactions. Abnormal SAHH expression may cause general alterations in cellular DNA methylation patterns and is known to alter various cellular characteristics (*see* Wolos *et al.*, 1993, *J. Immunol.* 150: 3264-3273; Liu *et al.*, 1992, *Antivir. Res.* 19: 247-265; Duerre *et al.*, 1992, *Biochim. Biolog. Cellulaire* 70: 703-711). The SAHH-derived cDNA insert from this experiment was re-cloned into the pLNCX vector in the same orientation as in the original provirus (*i.e.*, in the sense orientation) and used for further testing as described below.

The insert from the second transforming virus preparation was found to contain two different linked cDNA fragments, connected on one another by the adaptor. One of these fragments was derived from a cDNA encoding a structural

protein, filamin. The sequence of the other fragment, termed Tr19-GSE (shown in Figure 12) had no significant homology with any known genes in the NCBI database. These two fragments were re-cloned separately into the pLNCX retroviral vector for further testing.

Each of the re-cloned cDNA fragments were tested by transfection into ecotropic packaging cells and the resulting virus used to infect NIH 3T3 cells (to test for morphological transformation capacity for each cDNA insert) and MEF cells (to test for both immortalization and morphological transformation capacities). The NIH 3T3 cell experiments produced highly variable results. The MEF cell experiments, on the other hand, were more efficient and reproducible, and the results of these experiments are shown in Figures 13A-13C. Infection with virus carrying SAHH cDNA sequences (SAHH-GSE) resulted in both immortalization and morphological transformation of MEF cells. Infection with virus carrying the filamin cDNA fragment had no effect on MEF cells, but the Tr19-GSE-carrying virus was found to be capable of inducing immortalization of MEF cells, although at a lower efficiency than the SAHH-GSE. These results confirmed that the strategy disclosed herein had resulted in the isolation of two transforming GSEs, one of which was previously unknown (Tr19) and the other derived from a gene which, although known, had not been implicated in neoplastic transformation until now.

C. Selection of GSEs Enabling Tumorigenic Growth in Nude Mice

The following experiments were performed to isolate GSEs capable of enabling tumorigenic growth of NIH 3T3 cells in immuno-incompetent, nude (*nu/nu*) mice. The scheme for these experiments is shown in Figure 14. For this selection, RFRL-infected NIH 3T3 cells, prepared as described above, were inoculated subcutaneously into the flank of nude mice (Balb/c strain), at 5 x 10^5 cells per mouse. NIH 3T3 cells infected with pLNCX-vector derived virus were used as a control. Mice were examined weekly for tumor formation for up to six weeks post-inoculation. The results of these experiments are summarized in Table II.

TABLE II

	Number of Tumor-bearing Mice				
Cell Type	Week 2	Week 3	Week 4	Week 5	Week 6
Control	0/3	0/3	0/3	1/3	1/3
RFRL	0/9	6/9	7/9	9/9	9/9

These results, showing a higher frequency of tumorigenic variants among the NIH 3T3 cells infected with the RFRL-derived retrovirus than the LNCX-derived retrovirus, indicated the existence of tumorigenic GSEs in the population of RFRL-derived retroviruses. When the tumor size reached 5mm in diameter, each tumor was explanted and established in culture. PCR analysis performed using genomic DNA from three of these tumor-derived cultures showed the presence of several proviruses carrying different cDNA inserts. Virus was then rescued from these tumor cells by fusion of the tumor cells with ecotropic packaging cells, as described above, infection of fresh NIH 3T3 cells and selection in nude mice for tumorigenicity. Two mice were used per each transduced cell population, and proviral inserts from tumors formed in these mice were characterized by PCR analysis (shown in Figure 15). In two of the three populations tested, a single insert was found to be enriched in the secondary tumors of both independently-injected mice. A different insert was detected in the secondary tumors of mice injected with cells infected with virus derived from the third original NIH 3T3 cell population.

Both of these putative tumorigenic GSEs were characterized by nucleotide sequencing and the sequences compared with known nucleic acid and protein sequences present in the NCBI database. One of the cDNA inserts, termed Tr22-GSE, was found to share no significant homology with any of the sequences in the database, and hence represents a fragment of a novel gene (this sequence is shown in Figure 16). The other cDNA insert, termed 1bb1-GSE, is a sense-oriented GSE that encodes 87 amino acids from the internal region of the mouse homolog of the human P120 nucleolar antigen of proliferating cells. The nucleotide sequence of this GSE is shown in Figure 17, and nucleic acid and amino acid sequence comparisons between the P120 sequence and the GSE sequence are shown in Figures 18 and 19, respectively.

The 1bb1 fragment was re-cloned intro the pLNCX vector, transfected into ecotropic packaging cells, and the resulting virus used to infect Swiss 3T3 cells. Infection with the 1bb1-carrying virus resulted in the formation of morphologically-transformed foci in these cells (Figure 20). These results are consistent with a recent report that a full-length cDNA of P120 is capable of acting as a dominant oncogene in NIH 3T3 cells (Perlaky *et al.*, 1992, *Cancer Res.* 52: 428-436). The results disclosed herein indicate that the portion of the P120 cDNA comprising the 1bb1 GSE encodes a functional oncogenic domain representing about 10% of the P120 protein. This result is the first demonstration that such a small portion of an oncogenic protein is oncogenically functional.

EXAMPLE 3

Cloning And Analysis Of The Genes From Which Each Transforming GSE Was Derived

The results described in Example 2 above discloses the isolation of three newly-identified genes implicated in cellular transformation in tumor cells. Each of the genes corresponding to these three GSEs are isolated as follows. Each GSE is used as a hybridization probe to screen a mouse or human cDNA library prepared from normal cells. Interspecific DNA hybriudization at the appropriate stringency is expected to enable the isolation of genes corresponding to GSEs from any mammalian species, using nucleic acid probes that are homologous to GSEs or genes corresponding to such GSEs isolated as described in Example 2 above. The nucleotide sequence of the longest cDNA clone isolated in this way for each GSE is then determined, and the sequence analyzed to identify the longest open reading frame (ORF) encoding the putative gene product from each strand. Sequence homology analysis, as described above, is then performed on the sequence of the longest ORF to determine whether a related protein has been previously identified. If necessary, any additional nucleotides encoding amino acids from the amino terminus are then determined from 5'-specific cDNA isolated using the "anchored PCR" technique, as described by Ohara *et al.* (1989, *Proc. Natl. Acad. Sci. USA* 86: 5763-5677). Additional missing 3' terminal sequences are also isolated using this technique. The "anchored PCR" technique can also be used to isolate full-length cDNA starting directly from the GSE sequence without library screening.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Methods for Identifying Genetic Suppressor Elements and Genes Associated with Malignant Growth in Cancer Cells

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: PCT/US95/

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATCATCGAT GGATGGATGG 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCATCCATCC ATCGATGATT AAA 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTATGTAAC CCTGGCTATT CTGGAACTTG ATATCTAGAC CAGGCTGGCC TTGAACTCAA 60

ACAGATATCT TCCTGTTTCT GTCTCCTTAG TGCTGGGATA CAGTGTTTAG TGCTGCCATG 120

CTGGGTGGGA AGAGTATAAT AATAGCTCAT AGTTACTATG TTTGTTTAGG TTAGACATTT 180

TTTTTTCTGC TTTGTGTGTC TAATATGTTT GAACATCTCA TCTTCTTGAA ACTTGATGTG 240

GCTGTGTGAT TTGCTTTGGT TATTGAAAAG TGGCACATTG GCCAT 285

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 210 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACACGCCGT ACTTCCTCTG CTCAGCCCGT CTTTCCTCAT CATTGACCTT TTGTGTAGGC 60

AAGAGAACCC TCTGGGTGCA GTTTCATCTG CGGCTAAAGG ATCTCGCTGG CTCCGGTGGA 120

CCAGGTGAAA AGACACAGCT TTCTTCTTCT CTATAAAGGG CTTTTTCTTT CTGTGAGGCA 180

TAATGAGGCA GGGACACCCT CTCCGGAACC 210

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 273 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGATGATGGA GGTGGCAGCT GCCGATGTCC AGAGGCTGGG GGGCTCCGTG GAACTGGTGG 60

ATATCGGGAA GCAGAAGCTC CCAGATGGCT CGGAGATACC ACTTCTCCCA TCTGCTGGGC 120

AAGCTAGGCA GCGACCCCCA GAAGAAAACC GTGTGCATTT ACGGGCACCT GGACGTGCAG 180

CCTGCGCCCT GGAGGACGGG TGGGACAGCG AGCCCTTCAC CTTGGTGGAG CGGGAAGGCA 240

AGCTGTATGG GAGAGGCTCC ACGGACGATA AGG 273

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATTCACTGA GTTCATCAGT CCTAGCGGAA GCCGCCAGCA TGTCTGATAA ACTGCCCTAC 60

AAAGTCGCGG ACATCGGACT GGCCGCCTGG GGACGGAAGG CTCTGGATAT AGCTGAGAAT 120

GAGATGCCAG GGTTGATGCG CATGCGGGAG ATGTACTCAG CCTCCAAGCC ACTGAAGGGT 180

GCTCGCATTG CTGGCTGCCT GCGCATGACC GTGGAGACTG CTGTTCTCAT TGAGACTCTC 240

GTGGCCCTGG GTGCTGAGGC GCGGTGGTCC AGCTGCAACA TCTTC 285

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Ala Gln Pro Pro Ser Pro Val Ser Ile Thr Ser Ala Ala Ser Met
1 5 10 15

Ser Asp Lys Leu Pro Tyr Lys Val Ala Asp Ile Gly Leu Ala Ala Trp
20 25 30

Gly Arg Lys Ala Leu Asp Ile Ala Glu Asn Glu Met Pro Gly Leu Met
35 40 45

Arg Met Arg Glu Arg Tyr Ser Ala Ser Lys Pro Leu Lys Gly Ala Arg
50 55 60

Ile Ala Gly Cys Leu His Met Thr Val Glu Thr Ala Val Leu Ile Glu
65 70 75 80

Thr Leu Val Thr Leu Gly Ala Glu Val Gln Trp Ser Ser Cys Asn Ile
85 90 95

Phe

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 289 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCCCAGCCC CCTTCGCCCG TTTCCATCAC GAGTGCCGCC AGCATGTCTG ACAAACTGCC 60

CTACAAAGTC GCCGACATCG GCCTGGCTGC CTGGGGACGC AAGGCCCTGG ACATTGCTGA 120

GAACGAGATG CCGGGCCTGA TGCGTATGCG GGAGCGGTAC TCGGCCTCCA AGCCACTGAA 180

GGGCGCCCGC ATCGCTGGCT GCCTGCACAT GACCGTGGAG ACGGCCGTCC TCATTGAGAC 240

CCTCGTCACC CTGGGTGCTG AGGTGCAGTG GTCCAGCTGC AACATCTTC 289

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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His Ser Leu Ser Ser Ser Val Leu Ala Glu Ala Ala Ser Met Ser Asp
1               5                  10                  15

Lys Leu Pro Tyr Lys Val Ala Asp Ile Gly Leu Ala Ala Trp Gly Arg
            20                  25                  30

Lys Ala Leu Asp Ile Ala Glu Asn Glu Met Pro Gly Leu Met Arg Met
        35                  40                  45

Arg Glu Met Tyr Ser Ala Ser Lys Pro Leu Lys Gly Ala Arg Ile Ala
    50                  55                  60

Gly Cys Leu Arg Met Thr Val Glu Thr Ala Val Leu Ile Glu Thr Lys
65                  70                  75                  80

Val Ala Leu Gly Ala Glu Ala Arg Trp Ser Ser Cys Asn Ile Phe
                85                  90                  95
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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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CCCGGCCAAT CACCCTTCGG ACCAACACCT TGAAAACCCG TCGCCGAGAC CTTGCTCAGG      60

CTCTGATCAA TCGTGGGGTT AATCTGGATC CACTGGGGAA GTGGTCAAAG TCTGGACTTG     120

TGGTATATGA TTCTTCAGTG CCTATTGGTG CTACCCCTGA GTACCTCGCT GGACACTATA     180

TGCTGCAGGG AGCTTCCAGT ATGTTGCCCG TCATGGCCCT GGCACCTCAG GAGCATGAGC     240

GGATCTTAGA CATGTGCTGT GCT                                              263
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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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Lys Leu Met Asp Leu Phe Pro Leu Ser Glu Leu Val Glu Phe Leu Glu
1               5                  10                  15

Ala Asn Glu Val Pro Arg Pro Val Thr Leu Arg Thr Asn Thr Leu Lys
            20                  25                  30

Thr Arg Arg Arg Asp Leu Ala Gln Ala Leu Glu Asn Arg Gly Val Asn
        35                  40                  45

Leu Asp Pro Leu Gly Lys Trp Ser Lys Thr Gly Leu Val Val Tyr Asp
    50                  55                  60

Ser Ser Val Pro Ile Gly Ala Thr Pro Glu Tyr Leu Ala Gly His Tyr
65                  70                  75                  80

Met Leu Gln Gly Ala Ser Ser Met Leu Pro Val Met Ala Leu Ala Pro
                85                  90                  95

Gln Glu His Glu Arg Ile Leu Asp Met Cys Cys Ala
            100                 105
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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 262 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCGGCCCGT CACCCTCCGG ACCAATACCT TGAAAACCCG ACGCCGAGAC CTTGCACAGG 60

CTCTAATCAA TCGTGGGGTT AACCTGGATC CCCTGGGCAA GTGGTCAAAG ACTGGACTAG 120

TGGTGTATGA TTCTTCTGTG CCCATTGGTG CTACCCCCGA GTACCTGGCT GGGCACTACA 180

TGCTGCAGGG AGCCTCCAGC ATGTTGCCCG TCATGGCCTT GGCACCCCAG GAACATGAGC 240

GGATCCTGGA CATGTGTTGT GC 262

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Pro Val Thr Leu Arg Thr Asn Thr Leu Lys Thr Arg Arg Arg Asp
1 5 10 15

Leu Ala Gln Ala Leu Ile Asn Arg Gly Val Asn Leu Asp Pro Leu Gly
20 25 30

Lys Trp Ser Lys Thr Gly Leu Val Val Tyr Asp Ser Ser Val Pro Ile
35 40 45

Gly Ala Thr Pro Glu Tyr Leu Ala Gly His Tyr Met Leu Gln Gly Ala
50 55 60

Ser Ser Met Leu Pro Val Met Ala Leu Ala Pro Gln Glu His Glu Arg
65 70 75 80

Ile Leu Asp Met Cys Cys Ala
85

WE CLAIM:

1. A method of isolating genetic suppressor elements associated with establishment or maintenance of a transformed phenotype in a mammalian cell, the method comprising the steps of:

(a) synthesizing randomly fragmented cDNA prepared from the total mRNA of a cell to yield DNA fragments;

(b) transferring the DNA fragments to an expression vector to yield a genetic suppressor element library, wherein the expression vector is capable of expressing the DNA fragments in a living cell that is capable of expressing a transformed phenotype;

(c) genetically modifying the living cells by introducing the genetic suppressor element library into the cells;

(d) isolating or enriching for genetically modified living cells containing genetic suppressor elements conferring the transformed phenotype on the cells by selecting the cells under conditions wherein the transformed cells are identifiable;

(e) obtaining the genetic suppressor element conferring the transformed phenotype from the surviving genetically modified cells.

2. A genetic suppressor element identified by the method of claim 1.

3. A genetic suppressor element according to claim 2, wherein the genetic suppressor element is a sense oriented genetic suppressor element encoding a peptide.

4. A genetic suppressor element according to claim 2, wherein the genetic suppressor element is an antisense-oriented genetic suppressor element encoding an antisense RNA.

5. A synthetic peptide having an amino acid sequence corresponding to from about 6 amino acids to all of the amino acid sequence encoded by the GSE produced according to the method of claim 3.

6. A synthetic oligonucleotide having a nucleotide sequence from about 12 nucleotides to all of the nucleotide sequence of the antisense RNA encoded by the GSE produced by claim 4.

7. A cloned gene homologous to a nucleotide sequence of a GSE of Claim 2 or its complement.

8. The cloned gene according to Claim 7 wherein the GSE is selected from the group consisting of the GSEs Tr6, Tr19 and Tr22.

9. A mammalian cell that expresses a GSE according to claim 2.

10. A mammalian cell that expresses a GSE according to claim 3.

11. A mammalian cell that expresses a GSE according to claim 4.

12. An antibody raised against a protein encoded by, or a peptide encoded by a portion of, a gene of claim 7.

13. An antibody raised against a protein encoded by, or a peptide encoded by a portion of, a gene of claim 8.

14. A diagnostic assay comprising the steps of:

(a) isolating cellular RNA comprising messenger RNA from cancer cells from an animal;

(b) measuring a level of expression of an mRNA corresponding to a gene of Claim 7 in the cancer cells from the animal; and

(c) determining whether the level of expression of said mRNA measured in subpart (b) indicates that the gene is over-expressed or under-expressed in the cancer cells of the animal.

15. A diagnostic assay comprising the steps of:

(a) isolating cellular protein from cancer cells from an animal;

(b) measuring an amount of a protein corresponding to a gene of Claim 7 in the cancer cells from the animal; and

(c) determining whether the amount of said protein measured in subpart (b) indicates that the gene is over-expressed or under-expressed in the cancer cells in the animal.

16. A diagnostic assay comprising the steps of:

(a) measuring an amount of an enzymatic activity in cancer cells from the animal, said enzymatic activity being a property of a protein corresponding to a gene of Claim 7; and

(c) determining whether the amount of enzymatic activity measured in subpart (b) indicates that the gene is over-expressed or under-expressed in the cancer cells in the animal.

17. A diagnostic assay for characterizing a malignant tumor in an animal comprising the step of quantitating the level of expression of a gene measured according to Claims 14, 15 or 16.

18. The diagnostic assay of Claims 14, 15 or 16 wherein the gene is homologous to the nucleotide sequence or its complement of a GSE selected from the group consisting of SAHH, 1bb1, Tr6, Tr19 and Tr22.

19. A method of inhibiting malignant growth in cancer cells in an animal, the method comprising the step of expressing in the cancer cell a complete gene of Claim 7.

20. The method of Claim 19 wherein the GSE is selected from the group consisting of SAHH, P120, and the genes homologous to the GSEs Tr6, Tr19 and Tr22.

Figure 1

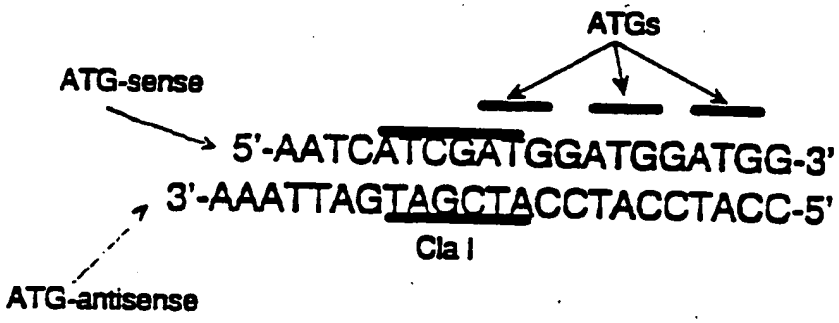
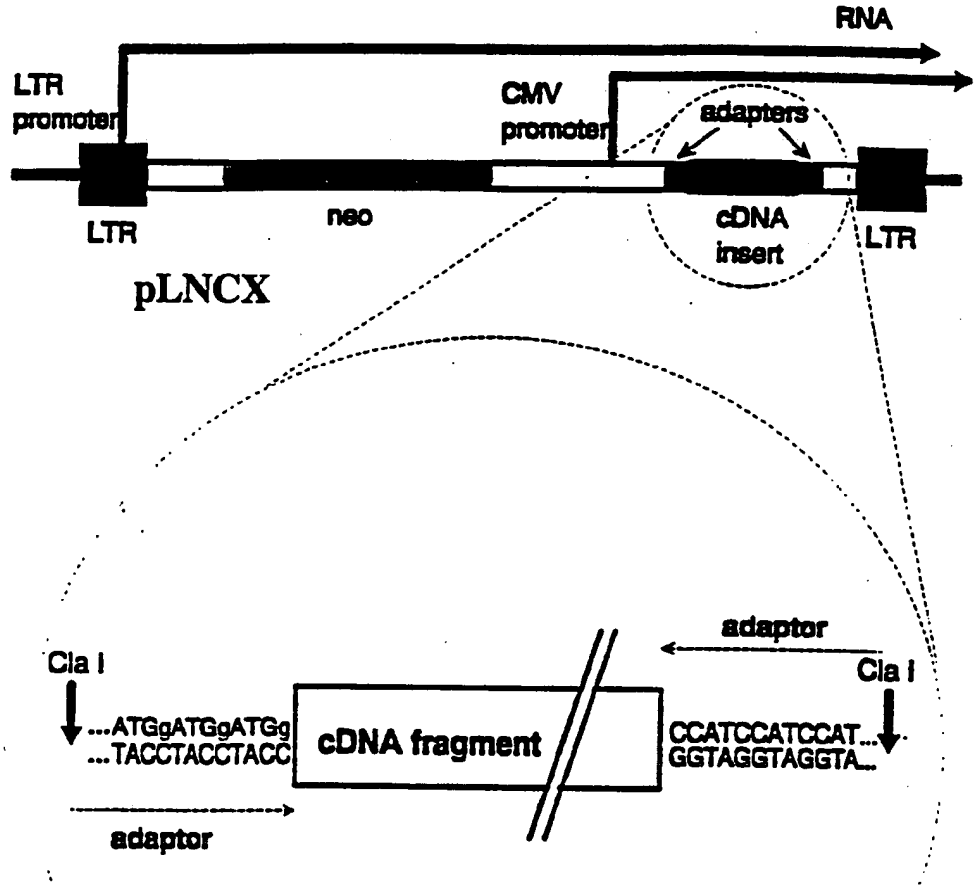

ATGs
ATG-sense
5'-AATCATCGATGGATGGATGG-3'
3'-AAATTAGTAGCTACCTACCTACC-5'
Cla I
ATG-antisense

Figure 2


RNA
LTR
promoter
CMV
promoter
adapters
LTR
neo
cDNA
insert
LTR
pLNCX
adaptor
Cla I
Cla I
...ATGgATGgATGg
...TACCTACCTACC
cDNA fragment
CCATCCATCCAT...
GGTAGGTAGGTA...
adaptor

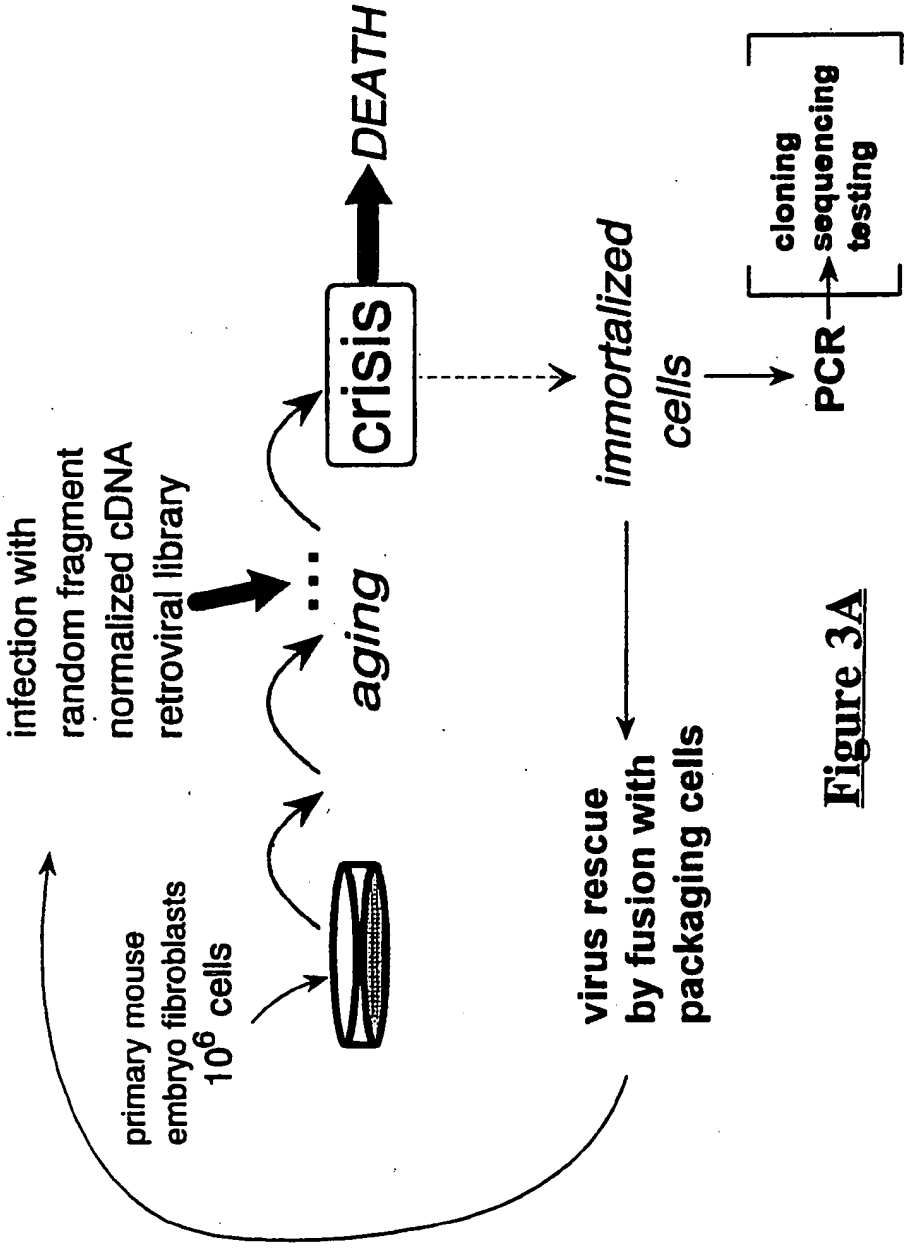
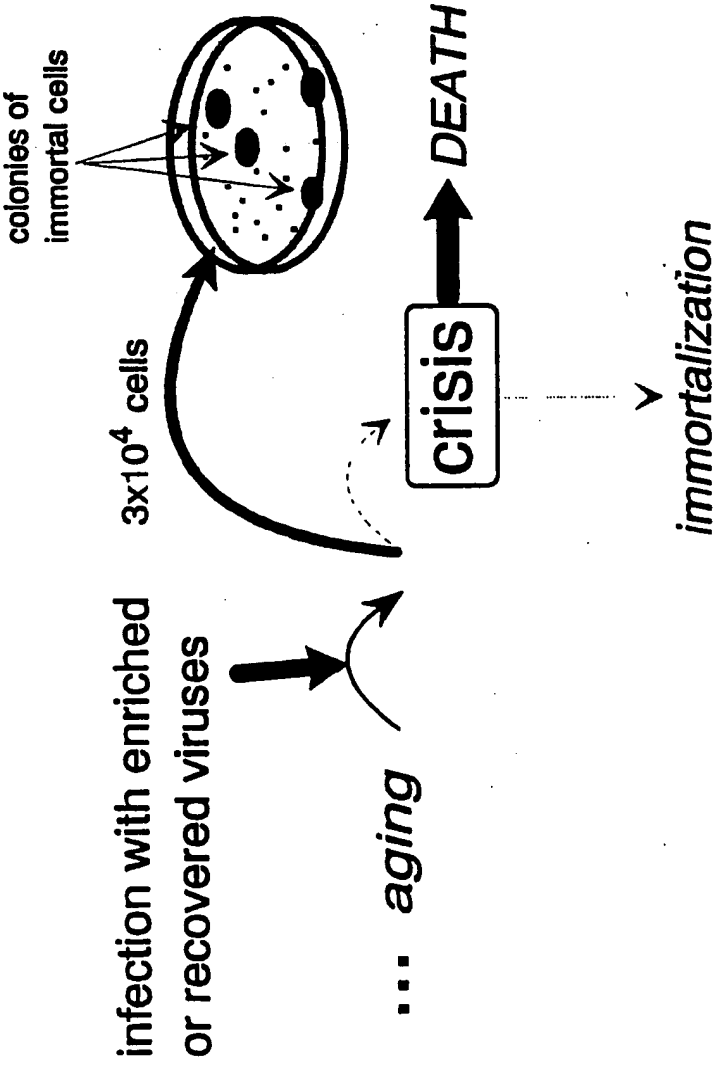

infection with
random fragment
normalized cDNA
retroviral library
primary mouse
embryo fibroblasts
10^6 cells
aging
crisis
DEATH
immortalized
cells
PCR
cloning
sequencing
testing
virus rescue
by fusion with
packaging cells

Figure 3A

Figure 3B


colonies of immortal cells
3x10^4 cells
infection with enriched or recovered viruses
... aging
crisis
DEATH
immortalization

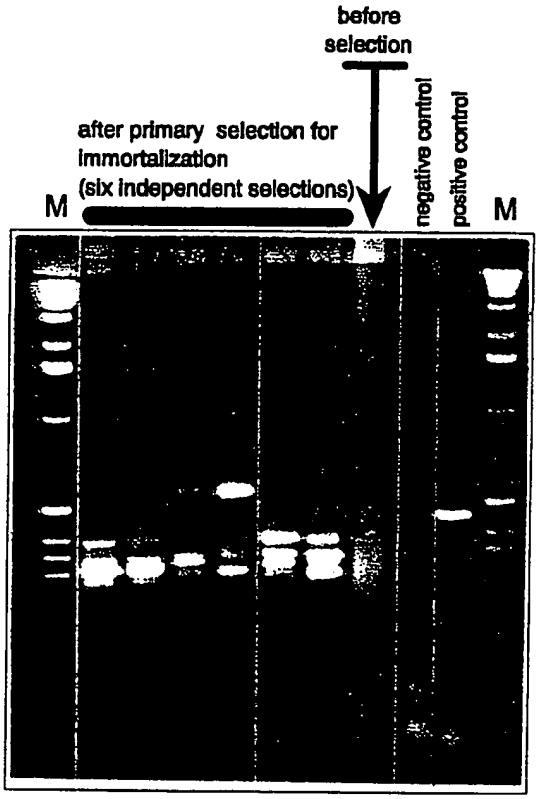

before
selection
after primary selection for
immortalization
(six independent selections)
negative control
positive control
M
M

Figure 4

```
GTTATGTAAC CCTGGCTATT CTGGAACTTG ATATCTAGAC CAGGCTGGCC TTGAACTCAA
ACAGATATCT TCCTGTTTCT GTCTCCTTAG TGCTGGGATA CAGTGTTTAG TGCTGCCATG
CTGGGTGGGA AGAGTATAAT AATAGCTCAT AGTTACTATG TTTGTTTAGG TTAGACATTT
TTTTTTCTGC TTTGTGTGTC TAATATGTTT GAACATCTCA TCTCTTTGAA ACTTGATGTG
GCTGTGTGAT TTGCTTTGGT TATTGAAAAG TGGCACATTG GCCAT
```

Figure 5

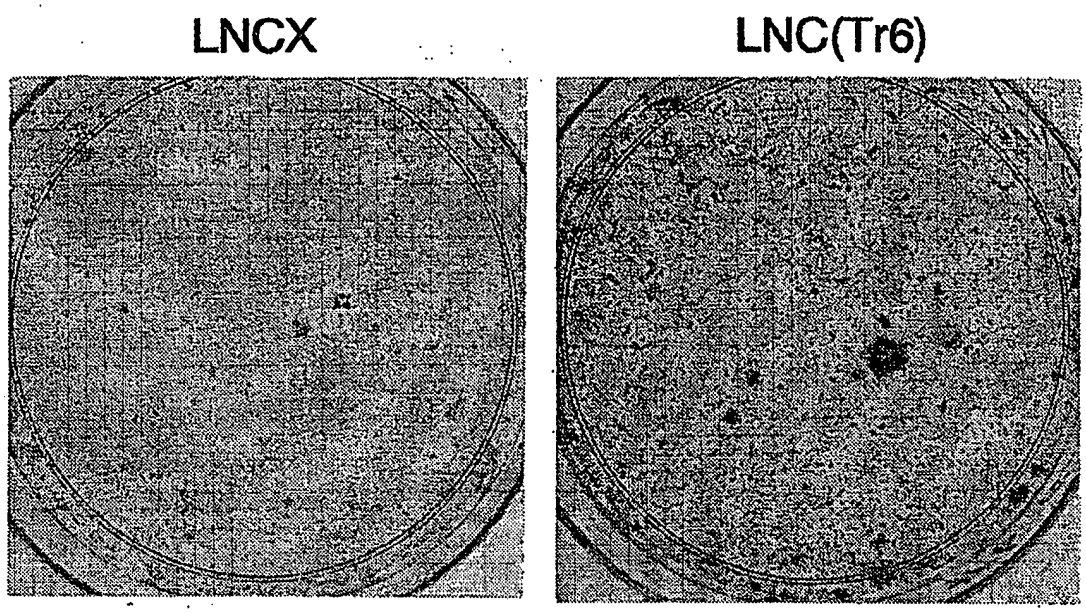


Figure 6A

Figure 6B

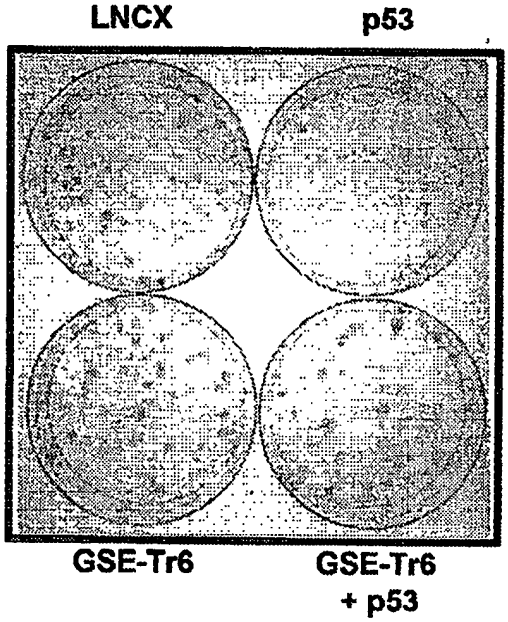
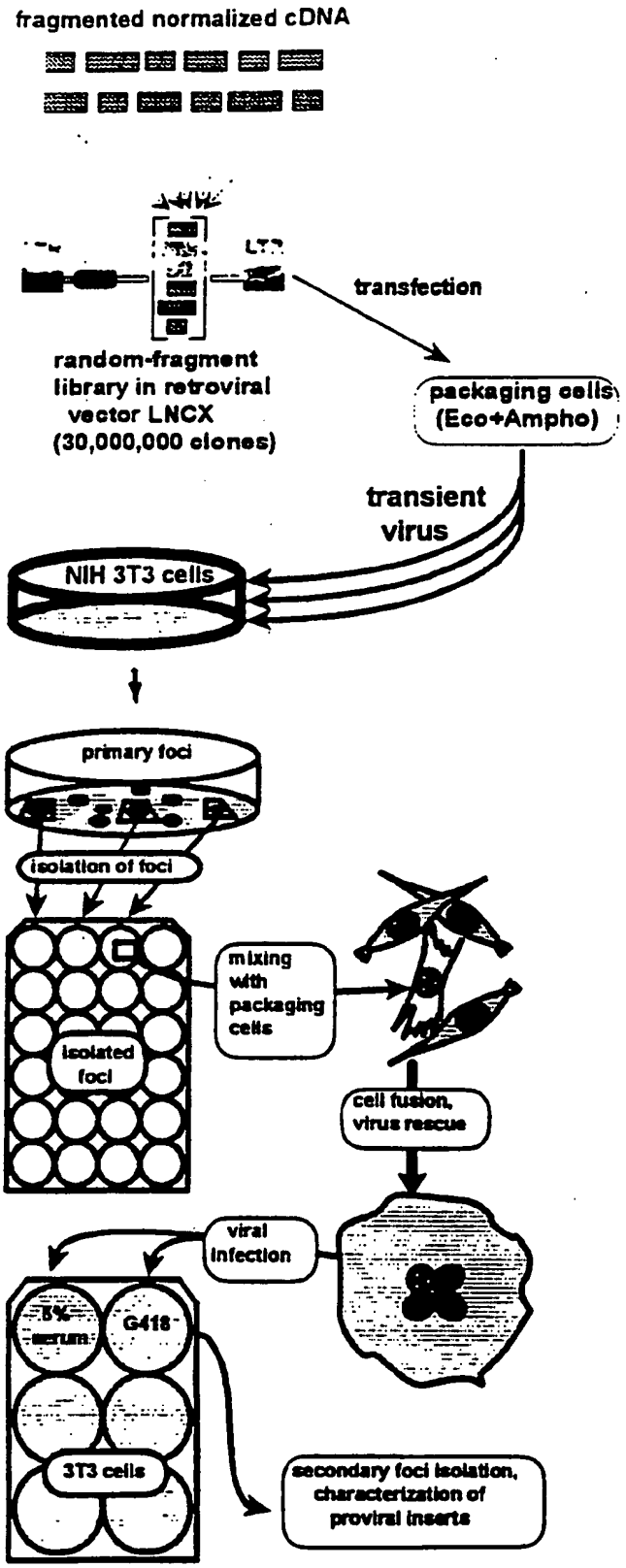


Figure 7


fragmented normalized cDNA
LTR
transfection
random-fragment library in retroviral vector LNCX (30,000,000 clones)
packaging cells (Eco+Ampho)
transient virus
NIH 3T3 cells
primary foci
isolation of foci
isolated foci
mixing with packaging cells
cell fusion, virus rescue
viral infection
5%
G418
3T3 cells
secondary foci isolation, characterization of proviral inserts

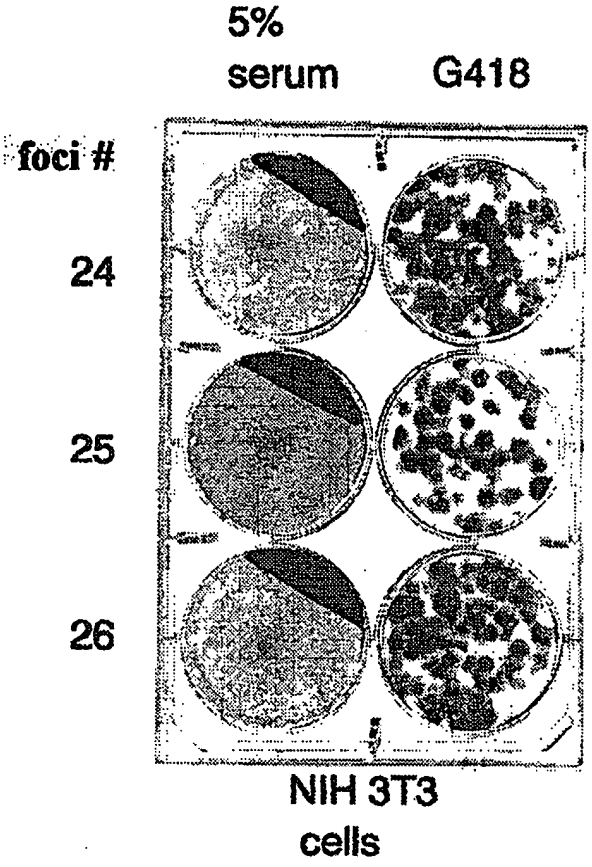


Figure 8A

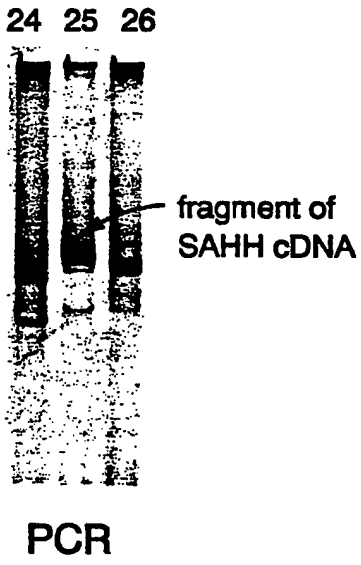


Figure 8B

Figure 9

```
CATTCACTGA GTTCATCAGT CCTAGCGGAA GCCGCCAGCA TGTCTGATAA ACTGCCCTAC
AAAGTCGCGG ACATCGGACT GGCCGCCTGG GGACGGAAGG CTCTGGATAT AGCTGAGAAT
GAGATGCCAG GGTTGATGCG CATGCGGGAG ATGTACTCAG CCTCCAAGCC ACTGAAGGGT
GCTCGCATTG CTGGCTGCCT GCGCATGACC GTGGAGACTG CTGTTCTCAT TGAGACTCTC
GTGGCCCTGG GTGCTGAGGC GCGGTGGTCC AGCTGCAACA TCTTC
```

Figure 10

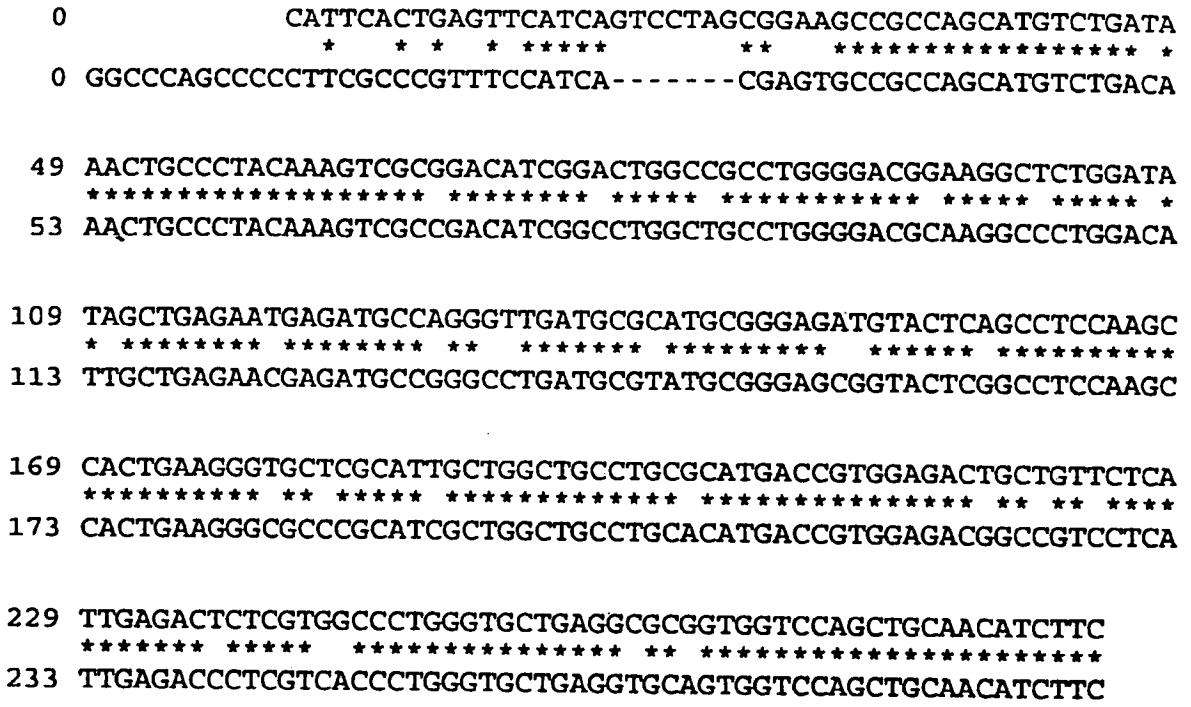

0 CATTCACTGAGTTCATCAGTCCTAGCGGAAGCCGCCAGCATGTCTGATA
0 GGCCCAGCCCCCTTCGCCCGTTTCCATCA-------CGAGTGCCGCCAGCATGTCTGACA
49 AACTGCCCTACAAAGTCGCGGACATCGGACTGGCCGCCTGGGGACGGAAGGCTCTGGATA
53 AACTGCCCTACAAAGTCGCCGACATCGGCCTGGCTGCCTGGGGACGCAAGGCCCTGGACA
109 TAGCTGAGAATGAGATGCCAGGGTTGATGCGCATGCGGGAGATGTACTCAGCCTCCAAGC
113 TTGCTGAGAACGAGATGCCGGGCCTGATGCGTATGCGGGAGCGGTACTCGGCCTCCAAGC
169 CACTGAAGGGTGCTCGCATTGCTGGCTGCCTGCGCATGACCGTGGAGACTGCTGTTCTCA
173 CACTGAAGGGCGCCCGCATCGCTGGCTGCCTGCACATGACCGTGGAGACGGCCGTCCTCA
229 TTGAGACTCTCGTGGCCCTGGGTGCTGAGGCGCGGTGGTCCAGCTGCAACATCTTC
233 TTGAGACCCTCGTCACCCTGGGTGCTGAGGTGCAGTGGTCCAGCTGCAACATCTTC

Figure 11

```
  0 EAQPPSPVSITSAASMSDKLPYKVADIGLAAWGRKALDIAENEMPGLMRMRERYSASKPL
    .. ..*  ....**********************************.*******
  0 HSLSSS--VLAEAASMSDKLPYKVADIGLAAWGRKALDIAENEMPGLMRMREMYSASKPL

 60 KGARIAGCLHMTVETAVLIETLVTLGAEVQWSSCNIF
    ********** ********** ***** .********
 58 KGARIAGCLRMTVETAVLIETLVALGAEARWSSCNIF
```

Figure 12

```
AACACGCCGT ACTTCCTCTG CTCAGCCCGT CTTTCCTCAT CATTGACCTT TTGTGTAGGC
AAGAGAACCC TCTGGGTGCA GTTTCATCTG CGGCTAAAGG ATCTCGCTGG CTCCGGTGGA
CCAGGTGAAA AGACACAGCT TTCTTCTTCT CTATAAAGGG CTTTTTCTTT CTGTGAGGCA
TAATGAGGCA GGGACACCCT CTCCGGAACC
```

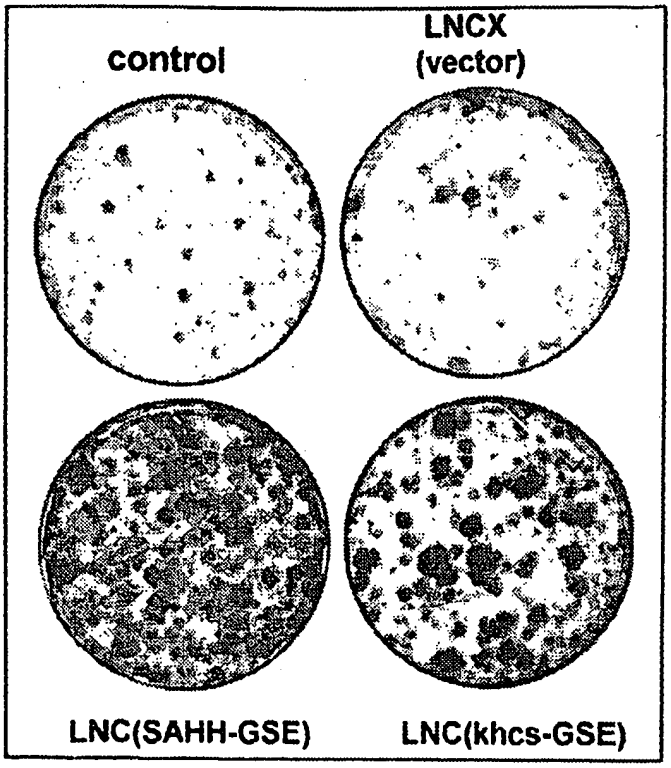


Figure 13A

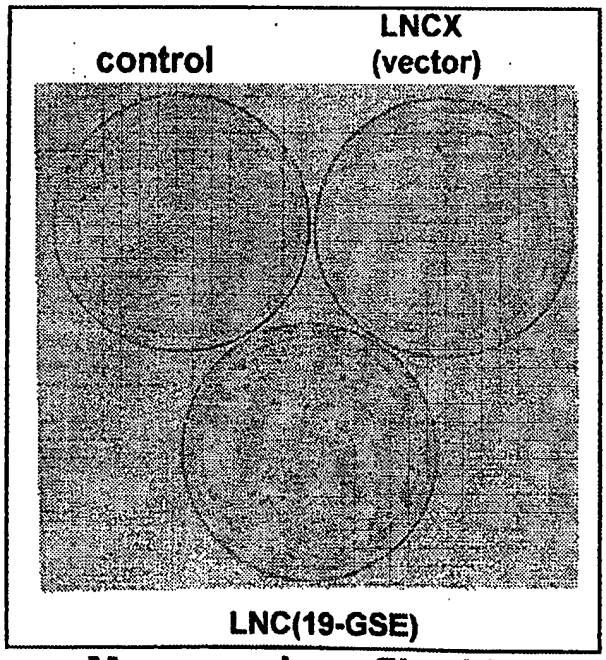


Figure 13B

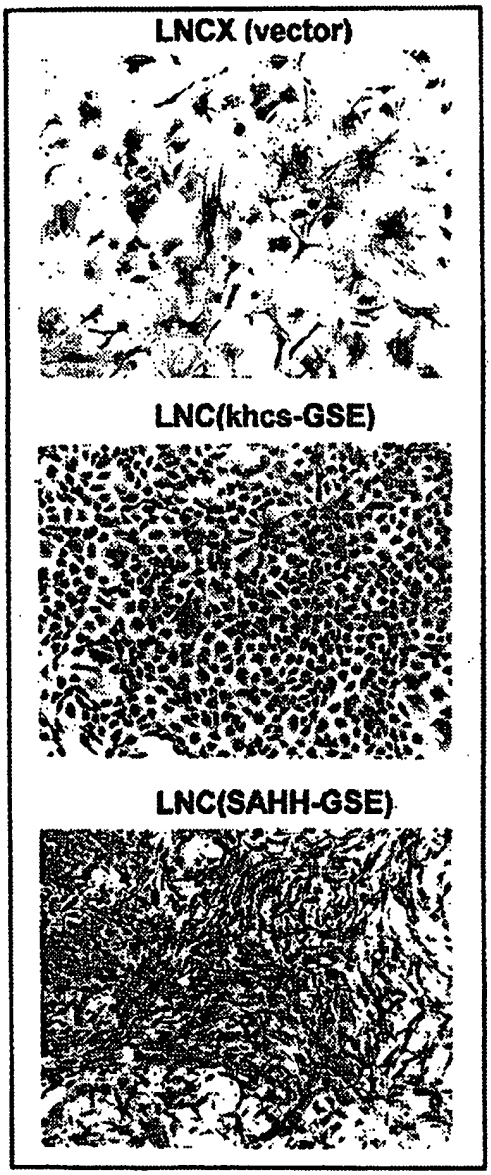

LNCX (vector)
LNC(khcs-GSE)
LNC(SAHH-GSE)
Mouse embryo fibroblasts

Figure 13C

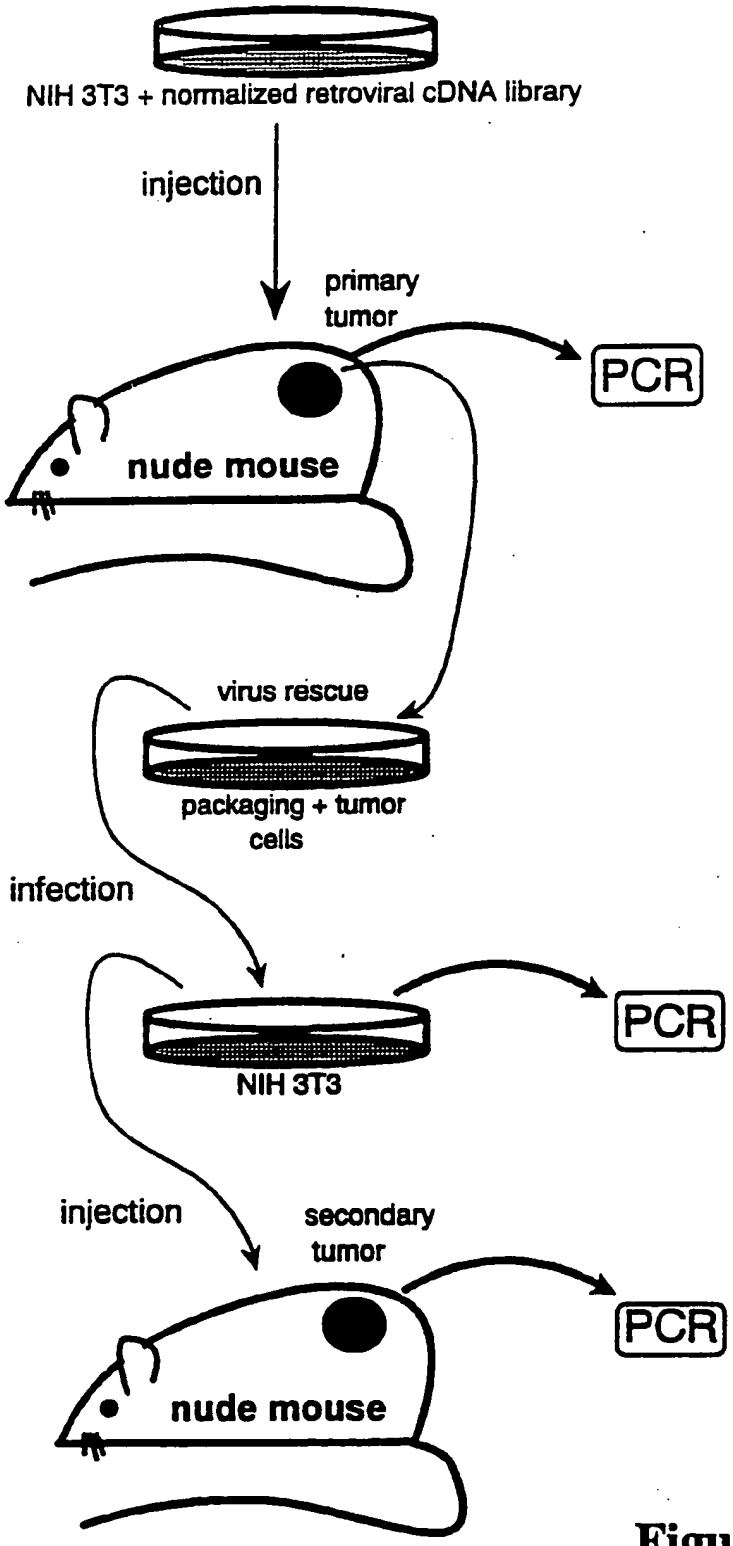

NIH 3T3 + normalized retroviral cDNA library
injection
primary tumor
PCR
nude mouse
virus rescue
packaging + tumor cells
infection
PCR
NIH 3T3
injection
secondary tumor
PCR
nude mouse

Figure 14

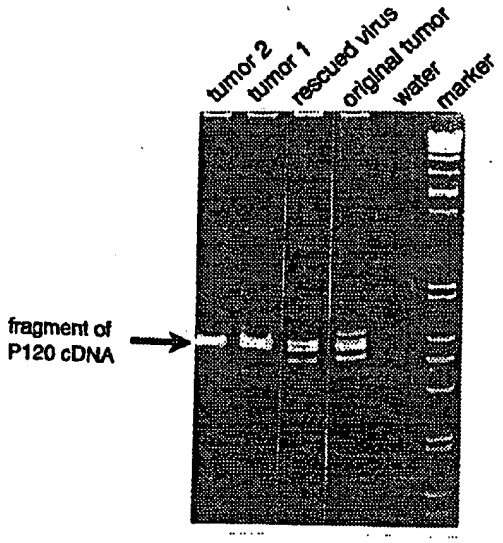

tumor 2
tumor 1
rescued virus
original tumor
water
marker
fragment of
P120 cDNA

Figure 15

Figure 16

```
GGATGATGGA GGTGGCAGCT GCCGATGTCC AGAGGCTGGG GGGCTCCGTG GAACTGGTGG
ATATCGGGAA GCAGAAGCTC CCAGATGGCT CGGAGATACC ACTTCTCCCA TCTGCTGGGC
AAGCTAGGCA GCGACCCCCA GAAGAAACC GTGTGCATTT ACGGGCACCT GGACGTGCAG
CCTGCGCCCT GGAGGACGGG TGGGACAGCG AGCCCTTCAC CTTGGTGGAG CGGGAAGGCA
AGCTGTATGG GAGAGGCTCC ACGGACGATA AGG
```

Figure 17

```
CCCGGCCAAT CACCCTTCGG ACCAACACCT TGAAAACCCG TCGCCGAGAC CTTGCTCAGG
CTCTGATCAA TCGTGGGGTT AATCTGGATC CACTGGGGAA GTGGTCAAAG TCTGGACTTG
TGGTATATGA TTCTTCAGTG CCTATTGGTG CTACCCCTGA GTACCTCGCT GGACACTATA
TGCTGCAGGG AGCTTCCAGT ATGTTGCCCG TCATGGCCCT GGCACCTCAG GAGCATGAGC
GGATCTTAGA CATGTGCTGT GCT
```

Figure 18

```
   0 CCCGGCCAATCACCCTTCGGACCAACACCTTGAAAACCCGTCGCCGAGACCTTGCTCAGG
     * *****  ******* ******** ************** ************** ****
 919 CTCGGCCCGTCACCCTCCGGACCAATACCTTGAAAACCCGACGCCGAGACCTTGCACAGG

  60 CTCTGATCAATCGTGGGGTTAATCTGGATCCACTGGGGAAGTGGTCAAAGTCTGGACTTG
     **** ***************** ******** ***** ************ ******* *
 979 CTCTAATCAATCGTGGGGTTAACCTGGATCCCCTGGGCAAGTGGTCAAAGACTGGACTAG

 120 TGGTATATGATTCTTCAGTGCCTATTGGTGCTACCCCTGAGTACCTCGCTGGACACTATA
     **** *********** ***** ************** ******** ***** ***** *
1039 TGGTGTATGATTCTTCTGTGCCCATTGGTGCTACCCCCGAGTACCTGGCTGGGCACTACA

 180 TGCTGCAGGGAGCTTCCAGTATGTTGCCCGTCATGGCCCTGGCACCTCAGGAGCATGAGC
     ************* ***** ****************** ******* ***** *******
1099 TGCTGCAGGGAGCCTCCAGCATGTTGCCCGTCATGGCCTTGGCACCCCAGGAACATGAGC

 240 GGATCTTAGACATGTGCTGTGC
     ***** * ******** *****
1159 GGATCCTGGACATGTGTTGTGC
```

Figure 19

```
  1                                    RPTTLRTNTLKTRRRDLAQALINRGVNLDPLGKWSKSGL
                                       ** .**********************************. **
300 KLMDLFPLSELVEFLEANEVPRPVTLRTNTLKTRRRDLAQALINRGVNLDPLGKWSKTGL

 39 VVYDSSVPIGATPEYLAGHYMLQGASSMLPVMALAPQEHERILDMCCA
    ********************************************
360 VVYDSSVPIGATPEYLAGHYMLQGASSMLPVMALAPQEHERILDMCCA
```

Figure 20